**PATENT IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re the application of:

Lasse LEINO et al.

Application Number: 10/534,988 Group Art Unit: 1612

Filed: May 16, 2005 Examiner: Isaac Shomer

For: PHARMACEUTICAL COMPOSITION FOR INTRACELLULAR ACIDIFICATION WITH CIS-UROCANIC ACID

## DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jarmo LAIHIA, declare as follows:

1. I am one of the inventors of the invention described and claimed in U.S. Patent Application S.N. 10/534,988 ("the application") and am aware the claims of the application have been rejected as unpatentable over Prater ("Immunotoxicity of Dermal Permethrin and Cis-Urocanic Acid: Effects of Chemical Mixtures in Environmental Health") in view of Wille et al. (U.S. Patent No. 5,843,979).

2. I am the Director of Research and Development for Bio-Cis Pharma Oy, the assignee of the application. I, or those

working under my supervision, performed experiments to measure

A. the metabolic activity and viability of non-stimulated neutrophils (polymorphonuclear leukocytes) from the human peripheral blood at pH 5.5 and pH 6.1, and

B. the inhibition of respiratory burst activity of stimulated neutrophils by cis-urocanic acid (cis-UCA) at pH 7.0 and pH 7.4.

A.1. Neutrophils were isolated from the peripheral blood of a healthy donor by conventional sedimentation techniques with high-molecular-weight dextran followed by density-gradient centrifugation without hypotonic lysis of residual erythrocytes to avoid unnecessary pH fluctuations in the cells. The isolated cells were washed and resuspended in Hank's Balanced Salt Solution containing 0.1% gelatin (gelatin-HBSS buffer). The cells, media, and centrifuges were kept at room temperature during cell preparation to avoid temperature fluctuations.

A.2. The effect of pH on the metabolic capability of neutrophils was studied by incubating an equal number of cells (100,000 in 0,18 ml of gelatin-HBSS buffer) at pH 5.5 and pH 6.1 in wells of a 96-well cell culture plate for 1 hour. The average metabolic rate of the cells was measured by using a colorimetric method (CellTiter 96 AQueous One Solution Cell Proliferation As-

say; Promega). This assay quantifies the formation of a formazan product from the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] in live cells. The measured end product absorbance correlates directly with the number of metabolically active cells in each well. As a second method, the number of viable cells was manually counted by trypan blue dye exclusion microscopy. This method distinguishes single live cells with an intact cell membrane from damaged dead cells that get stained with the marker dye.

A.3. In comparison to pH 6.1, incubation of the cells in a buffer solution adjusted to pH 5.5 reduced the number of metabolically competent cells by 46% ± 0.5% (mean ± sd of four replicate samples; $p < 0.001$, Student's t test) as measured by the colorimetric method as presented in Figure 1A below. The experiment was performed similarly with essentially identical results on three separate occasions ($p < 0.001$ in all experiments), each time using cells from a freshly drawn blood sample. The corresponding reduction in the number of viable cells was 57% (p value not calculated for a mean of two replicates analyzed) by the trypan blue method as presented in Figure 1B.

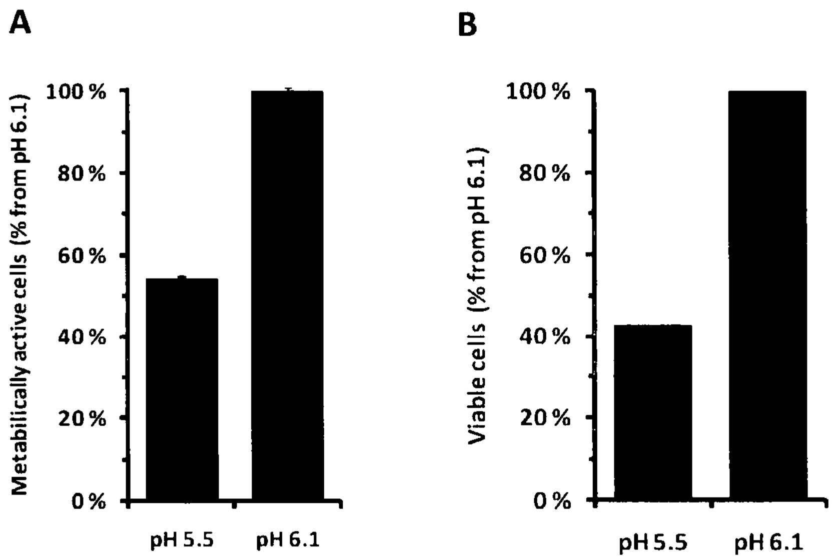


Figure 1. Effect of pH on the number of metabolically active (A; CellTiter 96 AQueous One Solution Cell Proliferation Assay; mean ± SD, n=4, $p < 0.001$) and viable cells (B; trypan blue dye exclusion assay; mean, n=2) of human neutrophils.

A.4. According to the obtained data, a change from pH 6.1 to pH 5.5 reduces the number of viable and metabolically competent cells by about 50%.

B.1. The effect of pH on the inhibition of respiratory burst activity of stimulated human neutrophils by cis-UCA was studied according to a methodology described in the application. Briefly, neutrophils were isolated from the peripheral blood of a healthy donor as explained above. An equal number of cells

(100,000 in 10 µl of gelatin-HBSS buffer was mixed with 150 µl of gelatin-HBSS buffer (pH 7.0 and pH 7.4) in quadruplicate wells of a 96-well cell culture plate and pre-conditioned at 37 °C for 5 min.

B.2. The respiratory burst reaction was initiated by adding 10 µl 10 mM luminol and 20 µl 400 nM phorbol 12-myristate 13-acetate (PMA) diluted in physiological saline in the wells. The intensity of chemiluminescence, indicating the speed of the respiratory burst reaction, was recorded at 36-second intervals in each well for the next 17-18 min. The experiment was performed similarly on three independent occasions, each time using cells from a freshly drawn blood sample.

B.3. The average respiratory burst activity (area-under-the-curve value with the blank value from unstimulated negative control cells subtracted) in control cells incubated at pH 7.0 in the absence of cis-UCA was equal or slightly higher than at pH 7.4. However, as shown in Figure 2 below, the average chemiluminescence in the presence of 10 mM cis-UCA was 14.7% from the control level at pH 7.0 and 29.3% from the control level at pH 7.4, indicating an about 50% enhancement in the potential of cis-UCA to inhibit respiratory burst activity at pH 7.0 in comparison to pH 7.4. In three independent experiments performed on separate occasions, each time using cells from a

freshly drawn blood sample, the inhibitory potential of 10 mM cis-UCA on respiratory burst activity of neutrophils was enhanced by 47.7% ± 0.5% (mean ± sd; $p < 0.001$, Student's t test) at pH 7.0 in comparison to pH 7.4 buffer.

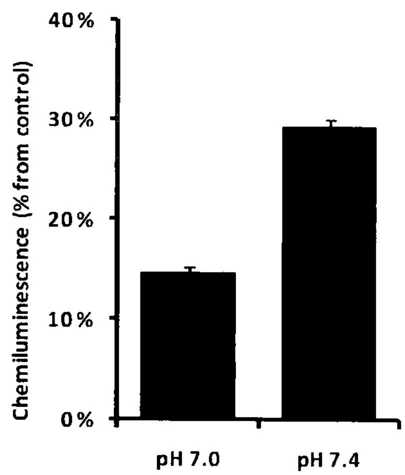


Figure 2. Inhibition of respiratory burst activity of stimulated human neutrophils by 10 mM cis-UCA at pH 7.0 and pH 7.4 (per cent from the corresponding pH control cells).

B.4. According to the obtained data, a change of the buffer from pH 7.4 to pH 7.0 increases the potential of 10 mM cis-UCA to inhibit the respiratory burst activity of PMA-stimulated human neutrophils by about 50%.

3. A. The MTT reduction assay is widely used for determining cell viability. There are three *in vitro* test systems based on the MTT method which have been validated by international regulatory authorities to identify the irritant ability of chemicals in the reconstructed human epidermis as replacement tests for *in vivo* skin irritation testing (OECD Guideline for the Testing of Chemicals, No. 439, In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, adopted 22 July 2010, p. 1, point 4; p. 2, point 12). The MTT assay involves a solubilization step where the insoluble end product is extracted in an organic solvent for measurement of the end product absorbance (ECVAM Skin Irritation Validation Study v. 1.8, EpiSkin™ Skin Irritation Test Method (15 min-42 hours), Standard Operating Procedure, Feb 2009, pp. 20-21, Ch. 5.9 and 6). The MTS reduction assay is an improved version of the MTT method, utilizing a chemically modified tetrazolium salt reagent which will be converted into a directly water-soluble end product by viable cells, and no solubilization step is required. The MTS assay is under a validation process by MatTek Corp. (Ashland, MA). The MTS assay is a commercial test product available from several manufacturers, and we have experience on this method for about ten years in our laboratory. We see no evidence to doubt the validity of the results shown above.

B. The trypan blue dye exclusion assay is a more traditional and well-established method for cell viability assessment. The number of viable cells are counted manually and divided by the total number of cells within the grids on a hemocytometer under microscope. Cells that take up trypan blue are considered non-viable. This method has been applied in a commercially available automated instrumental analysis of cell viability (Automated Viability Solutions, Beckman Coulter, Inc., p. 2, Trypan Blue Dye Exclusion Method). We see no evidence to doubt the validity of the results shown above.

4. A. The results suggest that the change of buffer pH from 6.1 to 5.5 causes a statistically highly significant reduction in the number of viable and metabolically competent neutrophils by about 50%. The acidic pH 5.5 environment is therefore significantly more harmful to living cells than pH 6.1. Because the MTT/MTS assay is able to predict the irritant ability of various compounds to live cells, the pH 5.5 is deduced to be more irritative than pH 6.1. Because the trypan blue exclusion assay is able predict the harmful effect of various compounds to the viability of live cells, the pH 5.5 is deduced to be more harmful than pH 6.1. As an extrapolation to the skin, a topical preparation adjusted to pH 6.1 or above will be significantly less irritative and less harmful to skin cell viability and metabolic

competence than a topical formulation with pH 5.5.

4. B. The results suggest that the change of buffer pH from 7.4 to 7.0 increases the inhibitory potential of cis-UCA on human neutrophil respiratory burst activity by about 50%. Respiratory burst is a phagocyte-specific cellular reaction directly reflecting the activity of the NADPH oxidase complex in the cell membrane. Upon activation, such as in inflammatory conditions in the skin, the NADPH oxidase complex of the inflammatory cells produces high amounts of superoxide which is a reactive oxygen radical species. Superoxide is instantly hazardous to local biological tissues, and it is also a precursor of other locally hazardous oxygen radicals. Tissue damage by reactive oxygen radicals will further aggravate and prolong the inflammatory reaction. We have shown in the application that cis-UCA is able to inhibit the respiratory burst activity of neutrophils by acidifying the cytosol of the cells. The present results confirm the observations presented in the application by showing that the ability of cis-UCA to inhibit the respiratory burst reaction is remarkably increased in the mildly acidic medium pH in contrast to neutral or alkaline pH. As shown here, the inhibitory potential of cis-UCA is statistically highly significantly enhanced at pH 7.0 in comparison to pH 7.4. As an extrapolation to the skin, a topical preparation containing cis-UCA, adjusted to pH

7.0 or below, will significantly enhance the potential of cis-UCA to inhibit the local release of oxygen radicals from inflammatory cells that infiltrate the inflamed skin. This will correspondingly decrease tissue damage, attenuate the inflammation reaction, and shorten the time of complete resolution of the skin inflammation. Thus, a cis-UCA-containing preparation adjusted to pH 7.0 or below is beneficial to the skin, compared to preparations with a pH above 7.0.

5. As a summarizing statement, the results presented in the present document confirm that administering cis-UCA in a composition with a carrier capable of adjusting the pH to the pH range 6.1-7.0 has surprising and non-obvious benefits. The lower pH 6.1 limit is justified by the unexpected finding that a preparation adjusted to pH 6.1 or above will be significantly less irritative and less harmful to skin cell viability and metabolic competence than a topical formulation with pH 5.5. The upper pH 7.0 limit is justified by the unexpected finding that a carrier adjusted to pH 7.0 or below will significantly enhance the potential of cis-UCA to inhibit the local release of oxygen radicals from inflammatory cells, as compared to a formulation with pH 7.4. Observing these two critical aspects that define the lower and upper limits of the pH range claimed in the application, admixing cis-UCA in a pharmaceutical composition adjusted

to the pH range 6.1-7.0 is based on surprising and unexpected findings. Although the claimed pH 6.1-7.0 range does overlap with the wide pH 1-12 range disclosed by Wille et al., narrowing the pH range of a pharmaceutical composition to pH 6.1-7.0 for the use of cis-UCA is not prima facie obvious for one of ordinary skill in the art. The selection of the claimed pH range 6.1-7.0 instead discloses an unpredictable inventive step that is not motivated by Prater or Wille et al. The claims of the application should therefore be accepted as patentable.

6. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. These statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Signed this 14 day of February, 2011.

Signed: [signature]

Name: Jarmo Laihia